The Lactococcus lactis CodY regulon - Identification of a conserved cis-regulatory element

den Hengst, C.D.; van Hijum, S.A F T; Geurts, J.M.; Nauta, A.; Kok, J.; Kuipers, O.P.

Published in:
The Journal of Biological Chemistry

DOI:
10.1074/jbc.M502349200

Publication date:
2005

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 40, pp. 34332–34342, October 7, 2005

The *Lactococcus lactis* CodY Regulon

IDENTIFICATION OF A CONSERVED cis-REGULATORY ELEMENT[*][S]

Received for publication, March 2, 2005, and in revised form, June 23, 2005 Published, JBC Papers in Press, July 21, 2005, DOI 10.1074/jbc.M502349200

Chris D. den Hengst[‡], Sacha A. F. T. van Hijum[‡], Jan M. W. Geurts[§], Arjen Nauta[§], Jan Kok[‡], and Oscar P. Kuipers[‡1]

From the [‡]Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, Kerklaan 30, 9751 NN Haren and [§]Friesland Foods Corporate Research, P. O. Box 87, 7400 AB Deventer, The Netherlands

CodY of *Lactococcus lactis* MG1363 is a transcriptional regulator that represses the expression of several genes encoding proteins of the proteolytic system. DNA microarray analysis, comparing the expression profiles of *L. lactis* MG1363 and an isogenic strain in which *codY* was mutated, was used to determine the CodY regulon. In peptide-rich medium and exponentially growing cells, where CodY exerts strong repressing activity, the expression of over 30 genes was significantly increased upon removal of *codY*. The differentially expressed genes included those predominantly involved in amino acid transport and metabolism. In addition, several genes belonging to other functional categories were derepressed, stressing the pleiotropic role of CodY. Scrutinizing the transcriptome data with bioinformatics tools revealed the presence of a novel overrepresented motif in the upstream regions of several of the genes derepressed in *L. lactis* MG1363Δ*codY*. Evidence is presented that this 15-bp *cis*-sequence, AATTTTCWGAAAATT, serves as a high affinity binding site for CodY, as shown by electrophoretic mobility shift assays and DNase I footprinting analyses. The presence of this CodY-box is sufficient to evoke CodY-mediated regulation *in vivo*. A copy of this motif is also present in the upstream region of *codY* itself. It is shown that CodY regulates its own synthesis and requires the CodY-box and branched-chain amino acids to interact with its promoter.

For the Gram-positive organism *Lactococcus lactis*, a lactic acid bacterium, auxotrophic for branched-chain amino acids (BCAAs),[2] methionine and histidine (1–3), maintenance of the nitrogen balance is essential. When this bacterium grows in milk, it uses the ubiquitous milk proteins (caseins) for its growth, employing a comprehensive and balanced proteolytic system (4–6). The extracellular cell wall-bound serine proteinase (PrtP) is an essential part of the proteolytic system, as it hydrolyzes the large caseins into smaller fragments. These peptides of various sizes, and free amino acids, can then be taken up into the cell by various transport systems, *e.g.* the oligopeptide transport system Opp (4) and the di- and tripeptide transport systems DtpP and DtpT (7, 8). Once inside, the peptides are degraded further by either endopeptidases (*e.g.* PepO and PepF) or aminopeptidases (*e.g.* PepN, PepX, and PepC) (9).

Although the proteolytic system of lactic acid bacteria has been characterized thoroughly over the past 20 years (6), new components are still being identified. For example, upon deletion of the main transport system for oligopeptides (*opp*), growth on media containing specific oligopeptides was still possible (10), suggesting the presence of at least one additional peptide transport system. Recently, a novel peptide transporter has been identified, encoded by *dpp* (*opt*), which is able to take over (part of) the role of *opp* (10–12).

Previous studies have shown that expression in *L. lactis* MG1363 of a number of genes of the proteolytic system is repressed in nitrogen-rich media. When *L. lactis* encounters limiting amounts of nitrogen, repression is relieved (13, 14). From recent studies it has become apparent that the transcriptional regulator CodY is responsible for repression of the genes *prtP/prtM*, *opp*, *pepN*, *pepC*, *araT*, and *bcaT* in response to nitrogen availability (14–17). More recently, it has been established that the nitrogen signal that affects the strength of repression by CodY consists of BCAAs. For both *L. lactis* and *Bacillus subtilis* it has been shown that these amino acids act as cofactors that directly stimulate CodY binding to regulatory sites of its target genes (17–19). An additional factor modulating the activity of CodY was identified in *B. subtilis*. In this organism, GTP, a marker of the energy state of the cell, can stimulate CodY binding by a mechanism independent of BCAAs. Such a mechanism seems to be absent in *L. lactis* (17, 18). Complex modulation of CodY activity in *B. subtilis* is probably required, as CodY exerts its effects on a wide variety of genes, among which are several that are not involved in nitrogen metabolism (20). CodY was first identified in this organism, where it serves as a nutritional repressor of the dipeptide-permease operon (21, 22) and of genes involved in amino acid metabolism (19, 23–28), carbon and energy metabolism (29), motility (30), antibiotics production (31), and competence development (20, 32).

Despite the detailed studies in *B. subtilis* and *L. lactis* on CodY-mediated regulation, which revealed direct interaction between CodY and the regulatory regions of its targets by means of *in vitro* DNA binding assays (17), no CodY recognition sequence could be deduced. Moreover, a genome-wide *in vivo* screening for CodY-DNA interaction sites in *B. subtilis* using a combination of chromatin immunoprecipitation and DNA microarray hybridization (ChIP-chip analysis) did not reveal any conserved sequences among members of the *B. subtilis* CodY regulon (20). It has been proposed that CodY recognizes and binds a three-dimensional structure formed by AT-rich DNA (22). CodY contains a C-terminal helix-turn-helix motif that is highly conserved in different bacterial species containing a homologue of CodY, and it might therefore be expected that these regulators recognize similar sequences in the respective host strains. In fact, it has been shown recently that lactococcal CodY is able to modulate the activity of a *B. subtilis* CodY target (18). To identify additional targets of *L. lactis* CodY and to assess whether this regulator is as pleiotropic as is its *B. subtilis* counterpart, DNA microarray experiments were carried out in which the transcriptional profile of

* This work was supported by Friesland Foods Corporate Research and the Dutch Ministry of Economic Affairs (SENTER). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1 and Tables 1–5.

[1] To whom correspondence should be addressed. Tel.: 31-50-363-2093; Fax: 31-50-363-2348; E-mail: O.P.Kuipers@rug.nl.

[2] The abbreviations used are: BCAA, branched-chain amino acid; EMSA, electrophoretic mobility shift assay; WT, wild type; IR, inversely repeated.

L. lactis MG1363 was compared with that of its *codY* mutant. By combining transcriptomics data with bioinformatics tools, a conserved motif in the regulatory regions of members of the *L. lactis* CodY regulon was identified. The importance of this DNA element for CodY-mediated regulation was investigated.

MATERIALS AND METHODS

Bacterial Strains, Media, and Preparation of Cells for RNA Isolation—L. lactis MG1363 and *L. lactis* MG1363Δ*codY* (TABLE ONE), which contains a 423-bp internal deletion in the *codY* gene (16), were grown at 30 °C in M17 broth (33) supplemented with 0.5% glucose (GM17). Cells were grown until the mid-exponential phase of growth (A_{600} ~1.0). Approximately 5×10^9 cells (50 ml of culture) were harvested by centrifugation for 1 min at 10,000 rpm and 20 °C. Cells were resuspended in 2 ml of ice-cold growth medium. After the addition of 500 μl of phenol/chloroform, 30 μl of 10% SDS, 30 μl of 3 M NaAc (pH 5.2), and 500 mg of glass beads (diameter 75–150 μm), cells were frozen in liquid nitrogen and stored at −80 °C until RNA isolation.

DNA Microarray Analysis—DNA microarray experiments were essentially performed as described (34). In short, RNA was isolated from four separately grown replicate cultures of *L. lactis* MG1363 and *L. lactis* MG1363Δ*codY*, prepared as described above. Subsequently, single-strand reverse transcription (amplification) and indirect labeling of 25 μg of total RNA, with either Cy3 or Cy5 dye, were performed (including a sample in which the dyes were swapped to correct for dye-specific effects) using the CyScribe post-labeling kit (Amersham Biosciences). Labeled cDNA samples were hybridized to slides representing 2110 open reading frames of *L. lactis* IL1403 spotted in duplicate and constructed as described (34, 35). After overnight hybridization, slides were washed for 1 min at room temperature in 2× SSC, 0.5% SDS and 5 min in 1× SSC, 0.25% SDS to remove nonspecifically hybridized cDNAs. Slides were scanned using a GeneTac LS IV confocal laser scanner (Genomic Solutions Ltd.). Subsequently, individual spot intensities were determined using ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, MD). Slide data were processed and normalized using MicroPrep (35, 36), which yielded average ratios of gene expression of mutant over wild type (WT) strain from the data of replicates. Expression of a gene was considered to be significantly altered when its ratio of expression in the mutant compared with the WT was >1.5 and had a p value <0.001 and false discovery rate <0.05 that were determined as described previously (35, 37). When a significantly up-regulated gene formed part of an operon, other members of that transcriptional unit were included, providing that they showed coregulation. A combined p value was calculated by multiplying their individual p values. All DNA microarray data obtained in this study are available online (www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc = GSE2823). In addition, the slide images and raw data are available at molgen.biol.rug.nl/publication/cody_data/.

Identification of DNA Motifs—To identify conserved DNA motifs, DNA sequences encompassing 200 bp of the upstream regions of the genes that were derepressed to the highest extent in the *codY* mutant in the DNA microarray experiments were collected from the genome sequence of *L. lactis* MG1363 (*L. lactis* MG1363 sequence information was contributed prior to publication[3] by Zomer *et al.*, and is available in supplemental Fig. 1). The regions upstream of *oppD*, *pepC*, *pepN*, *prtP*, and *prtM* were also included. This data set was used as input for the MEME software tool (38) to search for over-represented sequences. A graphical representation of the identified motif was obtained using Genome2D software (39). A string search for the occurrence of identified motifs was performed in the genome of *L. lactis* MG1363. Alternatively, GenBank™ files containing the entire genomes of *L. lactis* IL1403 (40), *B. subtilis* 168 (41) and *Streptococcus pneumoniae* R6 (42) were used as templates. Previously, a position-specific weight matrix of the over-represented motif was generated using Genome2D software (39), which was used to scan the genomic sequences. Results of all motif searches are available in supplemental Tables 1–5.

DNA Preparation, Molecular Cloning, and Transformation—Routine DNA manipulations were performed as described (43). Total chromosomal DNA from *L. lactis* was extracted as detailed previously (44). Minipreparations of plasmid DNA from *L. lactis* were made using the High Pure Plasmid isolation kit from Roche Applied Science. Restriction enzymes, T4 DNA ligase, and DNA polymerases were purchased from Roche Applied Science. PCR amplifications were carried out using either *Pwo* DNA polymerase for cloning purposes or TaqDNA polymerase for colony PCR. PCR products were purified with the High Pure PCR product purification kit (Roche Applied Science). Electrotransformation of *L. lactis* was performed using a Gene Pulser (Bio-Rad) as described earlier (45).

Combinations of oligonucleotides ctrA-Pfor with ctrA-Prev2, asnB-F with asnB-R, or codY-Pfor with codY-Prev (TABLE TWO) were used to amplify the upstream regions of *ctrA*, *asnB*, and *codY*, respectively, from chromosomal DNA of *L. lactis* MG1363. The PCR products were digested with XbaI and PstI and transcriptionally fused to the promoterless *Escherichia coli lacZ* gene in the integration vector pORI13 (46) digested with the same enzymes. The resulting plasmids, pORI::PasnB, pORI::PasnB, and pORI::PcodY, respectively, were made in *L. lactis* LL108 (47), isolated, and introduced into *L. lactis* MG1363 and *L. lactis* MG1363Δ*codY* by coelectroporation with pVE6007 (TABLE ONE), a plasmid that specifies a thermosensitive RepA protein that can drive pORI13 replication (48). DNA fragments derived from P*ywcC*, containing a perfect CodY-box spaced with 5 or 10 bp of its promoter, were obtained by PCR using combinations of oligonucleotides ywcC-end with ywcC-FboxP5 and ywcC-FboxP10, respectively. These fragments were digested with XbaI and EcoRI and transcriptionally fused to the promoterless *E. coli lacZ* gene into the reporter plasmid pILORI4 (49). The resulting plasmids, pIL::PywcCp5 and pIL::PywcCp10, respectively, were introduced into *L. lactis* MG1363 and *L. lactis* MG1363Δ*codY* by coelectroporation. Similarly, P*ywcC* variants containing a randomized CodY-box sequence were obtained using combinations of oligonucleotides ywcC-end with ywcC-FboxR5 and ywcC-FboxR10.

Determination of β-Galactosidase Activity—Overnight cultures of *L. lactis* grown in GM17 were diluted to 1% in 50 ml of the same medium containing 2.5 μg/ml of both erythromycin and chloramphenicol (Sigma) for the maintenance of pORI13 and pVE6007. Cell samples were collected by centrifugation, and β-galactosidase activities were determined as described previously (50).

In Vitro DNA Binding Assays—EMSAs were carried out essentially as described previously (17). In short, purified PCR products were end-labeled with [γ-^{32}P]ATP and polynucleotide kinase (Amersham Biosciences). Combinations of oligonucleotides dppP-Pfor with dppP-Prev, asnB-F with asnB-R, ctrA-Pfor with ctrA-Prev2, opp-1 with opp-3, thrA-Pfor with thrA-Prev, and argG-2 with argG-3 were used to prepare DNA probes that comprise the (putative) promoter sequences of *dppP*, *asnB*, *ctrA*, *oppD*, *thrA*, and *argG*, respectively (TABLE TWO). For labeling of the ywcC variants, the same fragments as those for cloning purposes were used. Binding reactions were carried out in 20-μl volumes containing 20 mM Tris-HCl (pH 8.0), 8.7% (v/v) glycerol, 1 mM EDTA (pH 8.0), 5 mM $MgCl_2$, 100–250 mM KCl, 0.5 mM dithiothreitol, labeled DNA fragment (3000 cpm), 1 μg of bovine serum albumin, 1 μg

[3] A. L. Zomer, U. Wegmann, M. O'Connell-Motherway, A. Goesmann, D. Van Sinderen, C. A. Shearman, M. J. Gasson, G. Buist, O. P. Kuipers, and J. Kok, manuscript in preparation.

TABLE ONE

Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or genotype	Source or Ref.
Strains		
L. lactis		
MG1363	Lac$^-$; Prt$^{-;}$ Plasmid-free derivative of NCDO712	68
MG1363Δ*codY*	MG1363 derivative containing chromosomal deletion in *codY*	16
LL108	Cm^r, MG1363 derivative containing multiple copies of the pWV01 *repA* gene in the chromosome	47
Plasmids		
pORI13	Em^r; integration vector, promoterless *lacZ*, Ori$^+$, RepA$^-$ derivative of pWV01	46
PILORI4	Eryr; pIL252 carrying the multiple cloning site and promoterless lacZ of pORI13	49
pVE6007	Cm^r; Temp^s replication derivate of pWV01	48
pORI::PasnB	Em^r; pORI13 carrying a 226-bp *asnB* promoter fragment amplified with primers asnB-F and asnB-R	This work
pORI::PcodY	Em^r; pORI13 carrying a 498-bp *codY* promoter fragment	This work
pORI::PctrA	Em^r; pORI13 carrying a 233-bp *ctrA* promoter fragment amplified with primers ctrA-Pfor and ctrA-Prev2	This work
pIL::PywcCp5	Em^r; pILORI4 carrying a 146-bp *ywcC* promoter variant P5	This work
pIL::PywcCp10	Em^r; pILORI4 carrying a 146-bp *ywcC* promoter variant P10	This work
pIL::PywcCr5	Em^r; pILORI4 carrying a 146-bp *ywcC* promoter variant R5	This work
pIL::PywcCr10	Em^r; pILORI4 carrying a 146-bp *ywcC* promoter variant R10	This work

TABLE TWO

Oligonucleotides used in this study

Name	Sequence (5′–3′)[a]
asnB-F	AACTGCAGGATTTAGCTGACTTTGGCTAC
asnB-R	GCTCTAGACTTCTATCGTTAAGTCAGATG
ctrA-Pfor	AACTGCAGGAAAGCACCAGAAGTACTGA
ctrA-Prev2	GCTCTAGACGATAAAGCTCAAAATCGGC
dppP-Pfor	GTCTAGCTTACGACTTAAAATC
dppP-Prev	CCCTCTTTCATGAATTGTGTC
gltA-Pfor	AACTGCAGGTTGTCAAAGGTGAAGCCATTC
gltA-Prev	GCTCTAGAGTAAAATTCACTAGGAATTTG
opp-1	GCTCTAGACACTCACTTGTTTTGCTTCC
opp-3	AACTGCAGTAAAACAATAATAAAAGCAG
argG-2	GCTCTAGAATCCACCTGAATATGCC
argG-3	CGGAATTCACTCTGCCATGGCTCCGC
serC-Pfor	AACTGCAGCGCCAAAGAAGTTGAATGGAAC
serC-Prev	GCTCTAGAGGAAGTACACTAGGTCCTGCAC
thrA-Pfor	GTCAGCATTTTCATGCTATCTTC
thrA-Prev	GAGTAGCTGATGCGAGTGATG
codY-Pfor	AACTGCAGGATCATTTTCGGATTGTC
codY-Prev	GCTCTAGACAAATCGGTCACTCCATC
codY-Fbox2	GAGTCTAAGCGGCCGCTGATTTATTGTTTTTCATG
codY-Rbox2	TAAATCAGCGGCCGCTTAGACTCTCAACAAAAAAAG
ywcC-end	GATCTCTAGATTAAGATACACGTTTAGTATAACCGCC
ywcC-FboxP5	TCGAATTCAATTTTCTGAAAATTTATTTTTGCTGAAAACGC
ywcC-Fbox10	TCGAATTCAATTTTCTGAAAATTATTAATATTTTTGCTGAAAAC
ywcC-FboxR5	TCGAATTCGTAATAATATTACATTATTTTTGCTGAAAACGC
ywcC-FboxR10	TCGAATTCGTAATAATATTACATATTAATATTTTTGCTGAAAAC

[a] Restriction enzyme sites are underlined.

of poly(dI-dC) (Amersham Biosciences), and varying amounts histidine-tagged CodY protein (H6-CodY), purified as described previously (17). After incubation at 30 °C for 15 min, protein-DNA complexes were separated in 4% polyacrylamide gels and run in TBE buffer at 100 V for 1 h, which were dried after electrophoresis and used for autoradiography (17).

DNase I footprinting analyses using purified H6-CodY was performed essentially as described previously (17). Reactions were performed in the absence of BCAAs. DNA fragments were prepared by PCR. Combinations of oligonucleotides serC-Pfor with serC-Prev, asnB-F with asnB-R, ctrA-Pfor with ctrA-Prev2, codY-Pfor with codY-Prev, and gltA-Pfor with gltA-Prev (TABLE TWO) were used to prepare DNA probes that comprise the (putative) promoter sequences of *serC*, *ctrA*, *codY*, and *gltA*, respectively. A DNA fragment of the *codY* upstream region containing a mutated CodY-box was obtained using a

TABLE THREE

Summary of transcriptome comparisons of *L. lactis* MG1363Δ*codY* and *L. lactis* MG1363

Transcriptional unit	Expression ratio[a,b]	Significance (*p* value)[c]	Description[d]
dppA, P, B, C, D, F	10.5	10^{-13}	Dipeptide transport system
gltD, (B), lysA	6.1	10^{-10}	Glutamate biosynthesis
ilvD, B, N, C, A, aldB	5.4	10^{-19}	BCAA biosynthetic operon
(hisC), Z, G, D, B, ymdC, hisH, A, F, I, K	5.3	10^{-22}	Histidine biosynthetic operon
ctrA	5.1	10^{-5}	Cationic amino acid transporter
oppD, F, B, C, A, pepO1	4.9	10^{-29}	Oligopeptide permease
asnB	4.4	10^{-4}	Asparagine synthetase
gltA, citB, icD	2.8	10^{-8}	Krebs trichloroacetic acid cycle enzymes
serC, A, B	2.4	10^{-4}	Serine phosphatase
ArcD1, C1, C2,	2.1	10^{-8}	Arginine catabolic pathway
amtB	2.0	10^{-4}	NrgA-like protein
YnaD, A, C	1.7	10^{-7}	ABC transporter
yndA	1.6	10^{-5}	Conserved hypothetical protein
bcaT	1.6	10^{-4}	BCAA aminotransferase
ydcG	1.6	10^{-5}	Transcription regulator
ynhA	1.6	10^{-3}	Hypothetical protein
ydgC	1.5	10^{-4}	Amino acid permease
yqaB, dapB	1.5	10^{-7}	Hypothetical proteins
pi120	1.5	10^{-4}	Putative dUTPase
FtsW1	1.5	10^{-4}	cell division protein FtsW

[a] Shown is the ratio of gene expression defined in the gene (in an operon) that shows the highest ratio (underlined). Genes shown in parentheses were not represented on the glass slides used in the experiment or contained an amplicon sequence that did not share homology to *L. lactis* MG1363 sequences.
[b] Ratio:expression in *L. lactis* MG1363Δ*codY* over that in *L. lactis* MG1363.
[c] For operons, a combined *p* value was calculated as described under "Materials and Methods."
[d] Possible gene function (40).

two-step PCR procedure. PCR products obtained with combinations of oligonucleotides codY-Pfor with codY-Rbox2 and codY-Prev with codY-Fbox2 were used as a template for a second PCR, using oligonucleotides codY-Pfor and codY-Prev.

RESULTS

Role of CodY in Global Gene Expression—To identify the genes that constitute the regulon of *L. lactis* CodY, the transcriptional profile of *L. lactis* MG1363 was compared with that of *L. lactis* MG1363Δ*codY*, a strain containing a 423-bp internal deletion in *codY* (16). *L. lactis* CodY exerts strong repressing activity in rapidly growing cells in media containing excess nitrogen (15). Therefore, the cells used for the transcriptional analyses were cultured in GM17 medium and harvested in the exponential phase of growth. Under these conditions, both strains grow similarly, although the mutant has a somewhat longer lag phase (data not shown). RNA samples were prepared from each strain and, following cDNA synthesis and labeling, hybridized to DNA microarrays. Analysis of the DNA microarray data of four biological replicates revealed that the expression of ~30 genes was significantly influenced by the *codY* mutation (TABLE THREE). Of these, the only gene of which the transcript level did decrease significantly was, as expected, *codY* itself (data not shown).

As anticipated, the levels of transcription of the genes that constitute the *opp* operon were elevated in *L. lactis* MG1363Δ*codY*, as this operon has been identified previously as being repressed by CodY (13, 17). Although purified histidine-tagged CodY has been shown to directly interact with the upstream regions of the genes encoding the peptidases *pepN* and *pepC*, their expression levels were not significantly changed or only marginally elevated (below 1.5-fold), respectively, in the *codY* mutant (17).

The majority of the proteins specified by the derepressed genes fall into the functional category of amino acid transport and metabolism. The strongest derepressed transcriptional unit in *L. lactis* MG1363Δ*codY* from this category was the *opt* operon comprising *optSABCDF*. The coding regions of *opt* of *L. lactis* IL1403 are over 90% identical with those of *dpp* (*dppAPBCDF*) of *L. lactis* MG1363. As they have a similar genetic organization, *opt* is the *L. lactis* IL1403 counterpart of *dpp*, encoding a binding protein-dependent ABC transporter for dipeptides (11, 12). In fact, it has been shown recently that both transport systems have similar substrate specificities (10). As is the case for the known CodY targets, expression of *dpp*/*opt* is repressed in peptide-rich media and especially in media containing peptides with BCAA residues (11, 13). These observations demonstrate that *dpp*/*opt* is another member of the CodY regulon. The transcript level of *ctrA*, encoding a putative cationic amino acid permease, increased more than five times upon deletion of *codY*.

A different group of genes, the transcription of which was found to be elevated in *L. lactis* MG1363Δ*codY*, encompasses those involved in BCAA metabolism; the *ilv-ald* operon for BCAA biosynthesis (51, 52) was derepressed over five times. Thus, although *L. lactis* MG1363 like many other lactococcal dairy strains is auxotrophic for these amino acids, several genes of the BCAA biosynthetic operon are present on the chromosome and are actively transcribed (1, 53). The gene encoding the aminotransferase BcaT, catalyzing either the first step of aromatic or branched-chain amino acid catabolism or the last step of their biosynthesis (54, 55), was also significantly derepressed.

Transcription of a number of genes involved in the metabolism of certain amino acids, other than BCAAs, was also affected by the mutation in *codY*. In particular, members of the histidine biosynthetic *his* operon, and a number of genes specifying enzymes of the arginine deiminase pathway (56), were strongly derepressed in *L. lactis* MG1363Δ*codY*. Expression of the gene encoding a putative asparagine synthetase, *asnB*, was elevated over four times in the *codY* strain, as was

the case for *gltDB*, the product of which is predicted to catalyze the same reaction (57).

Another gene cluster not known previously to be controlled in *L. lactis* by CodY contains genes involved in the Krebs oxidative cycle (58). Expression of citrate synthase (*gltA*), isocitrate dehydrogenase (*icd*), and especially aconitase (*citB*) was derepressed in *L. lactis* MG1363Δ*codY*. These results suggest that, as is the case in *B. subtilis* (20, 29), CodY might be involved in regulation of both nitrogen and carbon metabolism in *L. lactis*.

Verification of DNA Microarray Results of asnB and ctrA Using lacZ Fusions—To validate part of the obtained transcriptome data, DNA fragments carrying the putative promoter regions of *asnB* and *ctrA* were transcriptionally fused to *lacZ* in the reporter plasmid pORI13 (46) and introduced into *L. lactis* MG1363 and *L. lactis* MG1363Δ*codY*. β-Galactosidase activity was monitored during growth in GM17, the same medium as was used in the DNA microarray experiments (Fig. 1*A*). Although the absolute β-galactosidase activities of the two constructs were rather different (the *lacZ* transcription level achieved by the P*asnB*-containing fragment was more than 25 times lower than that of the P*ctrA* fragment), their derepression in *L. lactis* MG1363Δ*codY* compared with the parent strain was similar (almost 10-fold for both). These results show that active promoter elements are present in the upstream regions of these genes and confirm the role of CodY in their transcriptional regulation.

Interaction of CodY with Several of the New CodY Targets—To distinguish whether repression of some of the newly identified targets by CodY occurs directly or indirectly, EMSAs were performed using purified H6-CodY (Fig. 2*A*). The EMSAs show that purified H6-CodY was capable of forming several protein-DNA complexes with radioactively labeled DNA fragments containing about 200 bp of the upstream regions of *dppA* (the first gene of the *dpp* operon), *asnB*, and *ctrA*, as was the case for the well studied *oppD*, *pepN*, and *pepC* promoters (17). These results indicate that CodY probably directly controls expression of these genes. Moreover, lactococcal H6-CodY also interacted with the putative regulatory regions of *gltA* and *serC*, as demonstrated below in DNase I footprinting experiments. In some of the DNA binding experiments, bands of lower electrophoretic mobility than that of the free probe were observed irrespective of the presence of H6-CodY. These bands probably correspond to a single-stranded probe, resulting from the high AT content of the DNA fragments used (17).

An Over-represented Motif Is Present in the Upstream Regions of a Number of the CodY-repressed Genes—Although interaction of CodY with several of its targets in both *B. subtilis* and *L. lactis* has been reported, a consensus CodY-binding site, if any, remained elusive. To assess whether the members of the *L. lactis* CodY regulon identified here might share such a sequence motif in their regulatory regions, an *in silico* sequence analysis was performed. A data set was generated containing only the upstream regions of the genes that were derepressed to the highest extent in the *codY* mutant (supplemental Fig. 1). We assumed that these genes are most likely to be under direct control of CodY and could, if it exists, contain a CodY-binding site. The upstream regions of *pepC* and *pepN*, which are known to be direct targets of CodY (17), were also added to the data set. In addition, the intergenic region of the divergently transcribed and plasmid-located *prtP* and *prtM* genes of *L. lactis* subsp. *cremoris* SK11 was included, as purified H6-CodY was shown to bind to this region in *in vitro* DNA binding studies (16). Because operator sites of regulatory proteins in bacteria are usually located close to their target promoters, fragments of 200 bp were chosen such that they encompassed the known or predicted promoter sequences of their cognate genes. In those cases where a complete

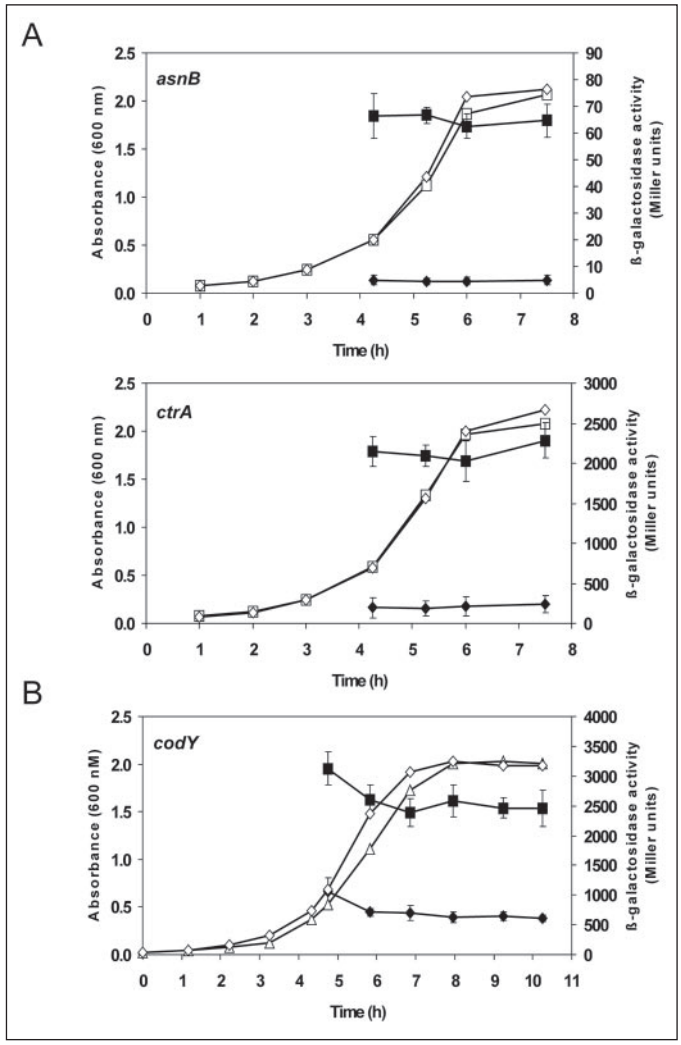

FIGURE 1. **Expression pattern of CodY-regulated genes.** *L. lactis* MG1363 and *L. lactis* MG1363ΔcodY, carrying fusions of the upstream region of *asnB*, *ctrA* (*A*), or *codY* (*B*), respectively, to *lacZ* in pORI13, were grown at 30 °C in GM17. β-Galactosidase activity was measured in time samples. Growth curves of *L. lactis* MG1363 (*open diamonds*) and *L. lactis* MG1363Δ*codY* (*open squares*) are shown as well as β-galactosidase activity in *L. lactis* MG1363 (*diamonds*) and *L. lactis* MG1363Δ*codY* (*squares*). The experiment was carried out in triplicate. *Error bars* indicate standard deviations.

operon was derepressed in the *codY* mutant, the upstream region of the first gene of the transcriptional unit was selected (*i.e.* for *ilv*, *his*, *opp*, and *dppAPBCDF*). Of the latter operon, the region preceding *dppP* was also included in the data set, because putative promoter elements are present in this area. This data set of 13 sequences was examined for the occurrence of common elements using the MEME algorithm (38). As there was no prior knowledge about a possible CodY-binding site, MEME was not restricted with respect to the motif width and the number of repetitions and was allowed to search on either of the two DNA strands. MEME was set to search only for inversely repeated (IR) sequences, as most DNA-binding proteins are known to bind sequences with such an organization. These parameters prevented common upstream elements (−35 and −10 sequences and the ribosome-binding site) from concealing the presence of possible CodY-binding sites. Application of the pattern recognition program revealed the presence of an over-represented motif in a number of the DNA sequences of the data set (Fig. 3) with homology to the upstream half-site of a palindromic sequence found to be important in *oppD* regulation (17). Derivates of this 15-bp IR *cis*-element, AATTTTCWGAAAATT, are present in the upstream regions

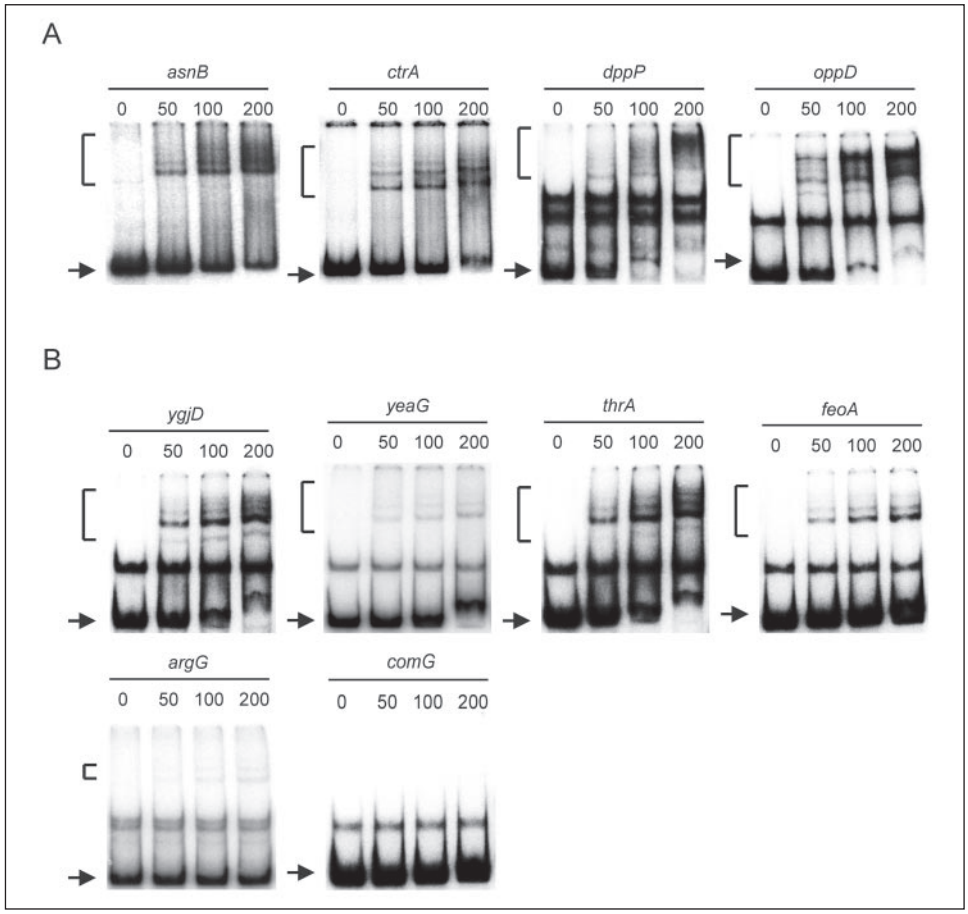

FIGURE 2. **Electrophoretic mobility shift assays using purified H6-CodY.** Probes were obtained by PCR and contain the upstream regions of CodY-regulated genes identified in DNA microarray analysis (*A*) and genes that contain a derivate of the CodY-box (*B*). Radioactively labeled probes containing ~200 bp of the upstream regions of *dppP*, *asnB*, *ctrA*, *oppD*, *thrA*, *ygjD*, *feoA*, or *yeaG* were incubated alone or with increasing amounts of H6-CodY (as indicated in nM above each lane). The positions of free probe and CodY-probe complexes are indicated in the *left margin* by *arrows* and *brackets*, respectively. Fragments containing the upstream region of *L. lactis argG* and *B. subtilis comG* served as negative controls.

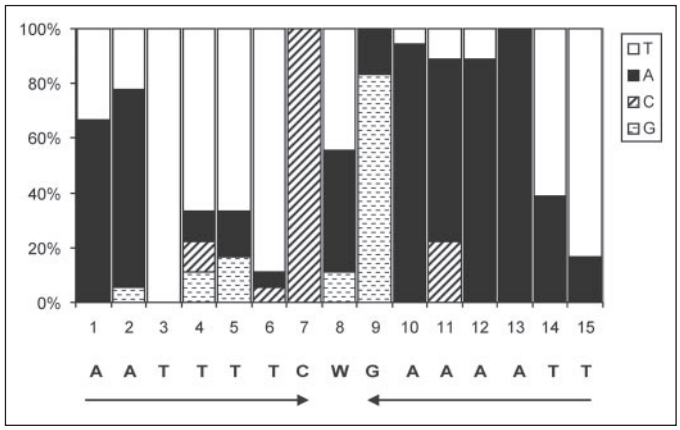

FIGURE 3. **Over-represented motif contained in upstream regions of CodY-regulated genes.** The conserved motif was identified using the MEME algorithm as described under "Materials and Methods." The weight matrix shows the percentage of A, C, T, or G nucleotides (as indicated in the legend) at each position of the motif. The inversely repeated consensus sequence (indicated by *arrows*) deduced from these frequencies is shown *below* the diagram where W can be an A or T nucleotide.

of 11 of the 13 genes that constitute the input data for the program (TABLE FOUR). A well conserved copy of this motif (designated CodY-box) seems to be absent from the upstream regions of *hisC* and *pepN*. Most interestingly, the upstream regions of the operons that show the highest fold difference in expression in *L. lactis* MG1363Δ*codY* compared with *L. lactis* MG1363 contain multiple copies of the CodY-box. Two copies of the motif are present close to *dppA*, *oppA*, and *ilvD*, whereas three copies can be discerned in the regions preceding *ctrA* and *gltA*.

To address whether copies of the CodY-box are present in the proximity of any of the other genes affected by the *codY* mutation (the ones that were not used to generate the data set for MEME) and in genes that were not affected in our transcriptome analysis, the entire genome of *L. lactis* MG1363 was searched for the occurrence of this motif using Genome2D software (39). By using a weight matrix (Fig. 3), derivates of the CodY-box were found throughout the lactococcal genome and were mainly located in intergenic regions. These were ranked according to their similarity with the consensus (supplemental Table 1). Obviously, high scoring motifs were found in the noncoding sequences that were used to build the weight matrix, but derivates of the motif were also found upstream of several other genes.

Purified CodY Binds to Sequences Containing a CodY-box in Vitro—To examine whether there is a relation between the occurrence of the CodY-box and the ability of CodY to bind DNA *in vitro*, DNase I footprinting experiments were performed using radioactively labeled fragments of about 250 bp encompassing the (putative) promoter regions of *gltA*, *serC,* or *ctrA* (Fig. 4). These fragments all contain well conserved copies of the CodY-box. Addition of purified H6-CodY protein resulted in protection against nuclease activity in one or multiple regions of all the probes, which indicates that *gltA*, *serC,* and *ctrA* are, most likely, direct targets of CodY. For all promoters, protected regions were observed that coincide with the nucleotide stretch formed by the CodY-box (Fig. 5).

To get an indication whether CodY might have a higher affinity for DNA that contains a CodY-box, EMSAs were performed using H6-CodY and DNA probes obtained by PCR amplification of a number of the regions encompassing the best scoring derivates of the CodY-box in *L. lactis* MG1363. Indeed, binding patterns similar to the ones pre-

sented in Fig. 2*A* were observed for all probes tested (Fig. 2*B*), except for the upstream region of *yeaG* for which H6-CodY was able to shift only a small fraction of the probes. H6-CodY was not able to bind to a negative control, an AT-rich DNA fragment containing the upstream region of the *B. subtilis comG* gene. Some H6-CodY binding occurred to the upstream region of *L. lactis argG*, whose transcription was found unaffected in the DNA microarray analysis and is not preceded by a CodY-box. However, H6-CodY poorly bound to this fragment and, compared with the other lactococcal probes tested, the fraction of shifted DNA was lowest. These results clearly demonstrate that the presence of a CodY-box can be used to predict whether a CodY-DNA interaction can occur *in vitro*.

TABLE FOUR

Conserved sequences in upstream regions of members of the *L. lactis* MG1363 CodY regulon

Gene	Location[a]	Strand[b]	Sequence
ctrA	−30	+	AATTGTCTGACAATT
dppA	−62	−	ATTTTTCTGACAATT
gltA	−31	−	TATTTTCTGAAAATT
dppP	+21	−	TATTGTCAGAAAATT
serC	−32	+	AATTATCAGAAAATT
oppD	−77	+	AATGTTCAGAAAATT
dppA	+11	−	AATATTCTGAAAATT
ilvD	−39	+	AATGTTCTGACAAAT
asnB	−48	+	AATTTCCAGACAATT
dppP	−62	−	TGTTTTCTGAAAATT
gltB	−55	−	TATATTCTGATAATT
gltA	−5	+	AATTTTCGGAATAAA
ctrA	−98	−	ATTCGTCAGTAAATT
pepC	−32	−	AATTATCAAAAAAAT
ctrA	−75	−	TTTTTTCAAAAAAAT
prtP	−74	+	AATTTACAGATAAAA
gltA	−56	+	TATTTTCTAAAAAAA
ilvD	−86	−	ATTCATCGGAATAAT
Consensus			AATTTTCWGAAAATT

[a] The location of the presented sequence is given relative to the (putative) transcriptional start site, which was determined experimentally or deduced by searching for sequences resembling consensus promoter elements.
[b] Orientation of the conserved sequence relative to the (putative) transcriptional start site.

A CodY-box Serves as an Operator Site for CodY—Whether the presence of a CodY-box is sufficient to evoke CodY regulation was examined by introducing a copy of the motif in front of a gene that is not regulated by CodY. The gene *ywcC* was selected as a candidate from the DNA microarray data, because it was actively transcribed under the growth conditions applied, and its expression was unaffected by the *codY* mutation. Four different variants of the upstream region of *ywcC* were obtained by PCR. They contained either a perfect or a randomized copy of the CodY-box and were introduced either at 5 or 10 bp upstream of the putative promoter of *ywcC*, placing the motifs at opposite sides of the helix. The ability of CodY to bind to these *ywcC* promoter variants was first tested in EMSAs (Fig. 6*A*). Purified H6-CodY was able to form a stable protein-DNA complex with both variants containing the artificial CodY-box *in vitro*, but not with the fragments containing a randomized box.

To find out whether the difference in binding is reflected in *in vivo* regulation by CodY, the different *ywcC* promoter variants were fused to promoterless *lacZ* in pILORI4 and introduced into *L. lactis* MG1363 and *L. lactis* MG1363Δ*codY*. Expression of *lacZ* expression was monitored during the exponential phase of growth in nitrogen-rich medium (Fig. 6*B*). No derepression was observed in the *codY* strain containing the randomized box or the variant carrying the consensus CodY-box located 5 bp upstream of the −35 sequence. Expression of the reporter construct containing a perfect CodY-box located at 10-bp upstream of the −35 consensus was almost 10-fold increased in the *codY* mutant relative to that in the wild type strain. This ratio is comparable with the ratios obtained for *asnB* and *ctrA*, two of the most prominent targets of *codY* found in the DNA microarray experiments (TABLE THREE; Fig. 1). Thus, a CodY-box can serve as an independent functional motif responsive to CodY protein *in vivo*.

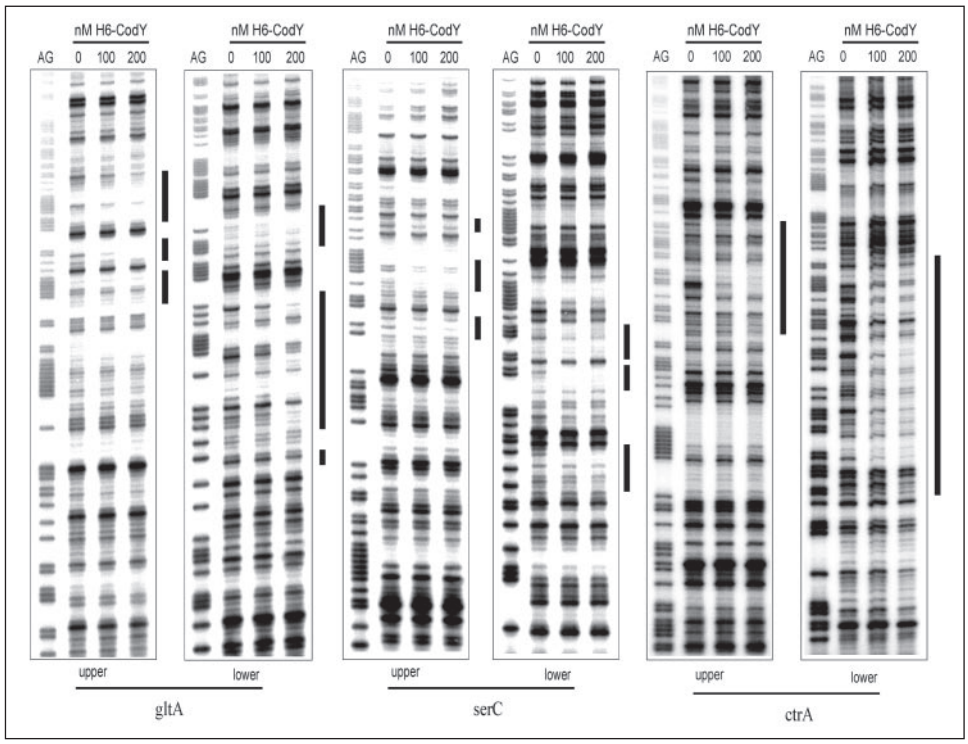

FIGURE 4. **DNase I footprinting analysis of H6-CodY binding to DNA.** Radioactively labeled probes containing ~200 bp of the upstream regions of *gltA*, *serC*, and *ctrA*, all containing a CodY-box, were examined alone or with different amounts of H6-CodY (as indicated above each lane) in DNase I footprinting analyses as described under "Materials and Methods." No BCAAs were added to the binding reactions. The *left* and *right halves* of each panel represent the footprint of the upper and lower DNA strand, respectively. Footprints are flanked on the *left* by Maxam and Gilbert A + G sequence ladders (*AG*). Protected regions are marked with *bars* in the *right margin*.

```
ctrA  CTGCATAAAGTTAATTCTTGACAATTGTCTGACAATTCGGTAAAATACAGTTTATTGAAATTATTTAGTTACAAATT-20
      GACGTATTTCAATTAAGAACTGTTAACAGACTGTTAAGCCATTTTATGTCAAATAACTTTAATAAATCAATGTTTAA

serC  TGTTTTTTTGTCGAACAGTATTTTTTTATATTTGAATTATCAGAAAATTATACTATAATAGTGATTAATAAATAATT-7
      ACAAAAAAACTGCTTGTCATAAAAAAATATAAACTTAATAGTCTTTTAATATGATATTATCACTAATTATTTATTAA

gltA  CTAAAAAAAAGTAATTTTAATTTTCAGAAAATATATAATTTTCGGAATAAAAAGTGTATAATAATGAAATAGATGGA+5
      GATTTTTTTTCATTAAAATTAAAAGTCTTTTTATATATTAAAAGCCTTATTTTTCACATATTATTACTTTATCTACCT

oppD  ATTTCGCGTAATGTTCAGAAAATTCATGAACATACCTAAAATAGTAAATTTTTGCAAATATGCAGAAAAAGTAGTAT-42
      TAAAGCGCATTACAAGTCTTTTAAGTACTTGTATGGATTTTATCATTTAAAAACGTTTATACGTCTTTTTCATCATA
```

FIGURE 5. **Summary of DNase I footprinting analysis of results presented in Fig. 4.** Protected bases are *underlined*. The position of the CodY-box is indicated in *boldface*. The (putative) −35 and −10 sequences are *overlined*. Numbers in the *right margins* of the upper strands indicate distances to the respective start codons of the genes indicated in the *left margin*. The *oppD* footprinting data were obtained previously (17).

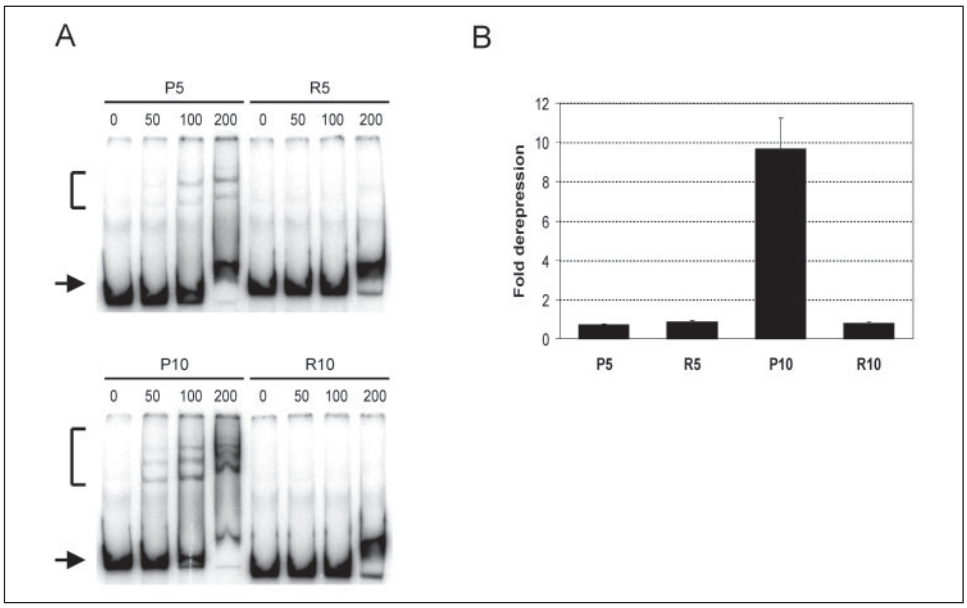

FIGURE 6. **H6-CodY binding and expression analysis of PywcC variants.** Radioactively labeled probes of PywcC variants were examined alone or with different amounts of H6-CodY (as indicated above each lane in nM) in EMSAs (*A*). Probes contain a perfect or a randomized copy of the CodY-box at 5 (P5 and R5, respectively) or 10 bp (P10 and R10, respectively) upstream of the promoter of PywcC. The positions of free probe and CodY-probe complexes are indicated in the *left margin* by *arrows* and *brackets*, respectively. *L. lactis* MG1363 and *L. lactis* MG1363Δ*codY*, carrying PywcC variants P5, R5, P10, and R10, respectively, fused to *lacZ* in pILORI4, were grown at 30 °C in GM17 (*B*). *Bars* indicate the ratio of β-galactosidase activity in the *codY* mutant over wild type MG1363 in cell samples harvested from the exponential phase of growth. The experiment was carried out in triplicate. *Error bars* indicate standard deviations.

The codY Promoter Region Contains a CodY-box and Is Auto-regulated—As a highly conserved copy of the CodY-box was found 86 bp upstream of *codY* itself and a CodY-box serves as an operator site for CodY, it could very well be that CodY regulates its own transcription. To examine this, a DNA fragment carrying the CodY-box and putative *codY* promoter sequences were transcriptionally fused to *lacZ* in the reporter plasmid pORI13 and introduced into *L. lactis* MG1363 and *L. lactis* MG1363Δ*codY*. β-Galactosidase activity was monitored during growth in nitrogen-rich medium (GM17), where high CodY activity is ensured (15, 17). In both strains, the expression driven by P*codY* was highest in the exponential phase and dropped to a lower (constant) level in the stationary phase of growth (Fig. 1*B*). Deletion of *codY* resulted in a 3–4-fold increase of β-galactosidase expression, indicating that CodY represses its own synthesis under these conditions. DNase I footprinting experiments revealed that CodY could interact with the upstream region of its own gene in a BCAA-dependent manner (Fig. 7), as the presence of at least one of the cofactors of CodY, Ile, was required. The region upstream of CodY that is protected by H6-CodY contains a CodY-box. H6-CodY did not protect a fragment in which the CodY-box was replaced by an unrelated sequence (CTAAGCGGCCGCTGA), irrespective of the presence of BCAAs, showing that the presence of a CodY-box is required for CodY binding (Fig. 7).

Presence of the CodY-box in Other Bacterial Species—To assess whether sequences homologous to the CodY-box are also present in other bacteria containing a CodY protein, a search was performed in the genomes of the Gram-positive bacteria *L. lactis* subsp. *lactis* IL1403, *B. subtilis* 168, and *S. pneumoniae* R6. If the CodY-box would also serve as an operator site for CodY in these organisms, such a comparison could reveal subtle differences in the consensus sequence between the various organisms. As shown in supplemental Tables 2–4, derivatives of the motif were identified in the genomes of *L. lactis* IL1403, *S. pneumoniae* R6, and in the upstream sequences of a number of the known CodY-regulated genes of *B. subtilis* (*e.g. hutP* and *dppA*). More interestingly, repression of the latter gene in a *B. subtilis codY* deletion strain could be complemented by *L. lactis codY*, indicating that lactococcal CodY can recognize similar sequences in *B. subtilis* (18). Recently, a genome-wide gene expression analysis of the *B. subtilis codY* mutant was reported (20). Using the upstream nucleotide sequences of the targets found in that study, we searched for the occurrence of the putative CodY-box in these sequences. Although the similarity scores with the consensus were not very high, derivates of the CodY-box were identified in some of these sequences, indicating that a similar motif might be operational in *B. subtilis* (supplemental Table 5).

As the *L. lactis* strains IL1403 and MG1363 are over 85% identical at the nucleotide level, CodY-box sequences were identified at similar positions in the *L. lactis* IL1403 genome. In the genome of *S. pneumoniae* R6, highly conserved copies of the CodY-box were found upstream of genes (putatively) involved in nitrogen metabolism and especially of those concerned with the biosynthesis of BCAAs. Most interestingly, the CodY-box is present upstream of *ppmA* and *rgg*, two genes that are known to be involved in virulence of this human pathogen (59, 60). In contrast to *B. subtilis*, a CodY-box is present in the upstream region of *S. pneumoniae* R6 *codY*, which suggests that transcription of *codY* of the latter organism, like that of *L. lactis*, is auto-regulated.

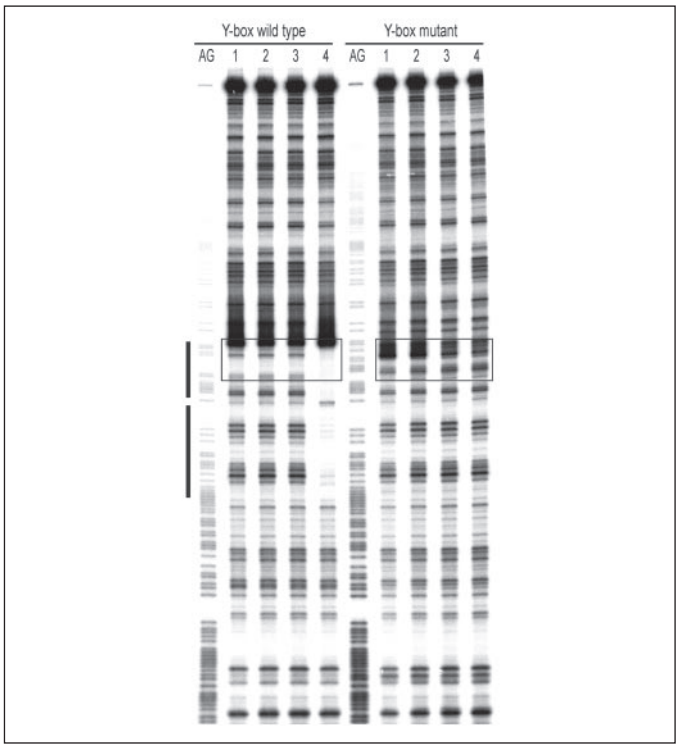

FIGURE 7. **DNase I footprinting analysis of H6-CodY binding to the *codY* promoter region.** A radioactively labeled probe containing the upstream region of *codY* was examined alone (*lane 1*) or in the presence of 120 (*lanes 2* and *4*) or 240 nM (*lane 3*) of H6-CodY in DNase I footprinting analyses as described under "Materials and Methods." The reactions shown in *lane 4* were performed in the presence of 7.5 mM Ile. The *left* and *right halves* of the panel show footprinting reactions using probes containing a wild type and a mutated CodY-box (regions enclosed by *boxes*), respectively. Footprints are flanked on the *left* by Maxam and Gilbert A + G sequence ladders (*AG*). Protected regions are marked with *bars* in the *left margin*.

DISCUSSION

In the last few years, knowledge about the pleiotropic regulator CodY has expanded rapidly. A large number of genes in *B. subtilis* have been shown recently to be regulated by this transcriptional repressor (20). The current study defines the regulon of *L. lactis* CodY and shows that deletion of the regulator has global effects on gene expression. In addition to the known members of the lactococcal CodY regulon, which are all involved in the degradation of casein and in peptide and amino acid uptake and metabolism, the newly identified genes also predominantly belong to this category. Apparently, when the cells reach the stationary phase and nutrients become scarce, CodY-mediated repression of peptide and amino acid transport systems is relieved to maintain the intracellular nitrogen balance. As the major peptide uptake systems are fully derepressed in *L. lactis* MG1363Δ*codY*, intracellular nitrogen pools are probably severely altered. The expression of genes (putatively) involved in the metabolism of a number of amino acids other than BCAAs, especially of genes connected to asparagine, glutamate, and histidine biosynthesis and of those required for arginine catabolism, was found to be strongly affected as well, which might be a way to counteract the effects of removal of *codY* on intracellular nitrogen availability.

Because of its broad effects on the proteolytic system and amino acid metabolism, it is rather surprising that the activity of lactococcal CodY seems to be modulated solely by BCAAs (15, 17, 18) and not by a more general signal of nitrogen availability. By using BCAAs as direct signaling molecules for CodY, *L. lactis* could ensure a proper supply of BCAAs, for which most dairy strains are auxotrophic (1). Moreover, BCAAs serve an important role in the synthesis of fatty acids and determine protein hydrophobicity. The central role of BCAAs in CodY-mediated regulation is reflected by the fact that the *ilv* operon is one of the strongest derepressed transcriptional units in *L. lactis* MG1363Δ*codY*, as was also the case in *B. subtilis* (19, 20, 27, 28). Although the lactococcal strain used in this study cannot synthesize all enzymes required for BCAA biosynthesis, due to nonsense mutations and small deletions in *ilv*, the encoding genes (including the downstream *aldB* gene) are still present, actively transcribed, and tightly regulated (1). As we show that this operon is one of major targets of CodY (TABLE THREE) and CodY-boxes are present in its upstream and coding regions (TABLE FOUR), CodY most probably directly controls *ilv* expression. Moreover, repression of this operon has been shown to be dependent on the BCAA Ile (51–53), which is the most potent modulator of CodY activity, as it has been shown that CodY binding to the promoter region of at least one of its targets, *oppD*, in DNA binding studies is stimulated most by this particular BCAA (17).

The effects of the deletion of *codY* in *L. lactis* were not restricted to genes involved in amino acid uptake and metabolism alone. In particular, expression of the *gltA-citB-icd* transcriptional unit, which encodes part of the Krebs oxidative (trichloroacetic acid) cycle, was altered. As these genes are actively transcribed in the strain used in this study, and a number of them are known to encode functional proteins (those constituting the oxidative branch) in related lactococci (61), our results suggest that *L. lactis* MG1363 CodY, like its *B. subtilis* counterpart, might be involved in regulation of both nitrogen and carbon metabolism (20, 29). The oxidative branch of the trichloroacetic acid cycle can provide the cell with α-ketoglutarate, which in turn is used in the formation of glutamate by some lactic acid bacterium (58, 62). In addition, α-ketoglutarate acts as a cosubstrate for the aminotransferase BcaT, which catalyzes the first step of BCAA catabolism (55). Thus, α-ketoglutarate provides a connection between BCAA and glutamate metabolism, which could explain why CodY orchestrates transcription of genes involved in the trichloroacetic acid cycle and glutamate biosynthesis (*gltDB* and *lysA*) in addition to those concerned with BCAA metabolism (*ilv* and *bcaT*). Although these genes are apparently linked at the transcriptional level, their biological role remains uncertain as some of the enzyme activities required for these reactions have not been determined in the lactococcal strain used in this study.

Expression of the gene encoding the aminotransferase BcaT has been shown recently to be controlled by CodY (54). The *bcaT* gene is repressed by CodY in a chemically defined medium supplemented with casitone, a complex source of nitrogen, and in that respect comparable with the GM17 used in this work (13, 33). Although expression of *bcaT* was significantly altered ($p < 0.001$) in our experiments as well, the extent of derepression measured on the DNA microarrays was somewhat low compared with that observed by Chambellon and Yvon (54). Changes in mRNA levels were lower for all the genes examined here for which CodY repression ratios have already been determined, yet the trends are comparable (13, 15–17). Compression of the observed differential expression becomes apparent from comparing Fig. 1 with the relevant data in TABLE THREE. This effect has been shown to be inherent to the DNA microarray methodology (63) and might explain why not all previously identified CodY targets were found in this study. Alternatively, it could be that these genes are regulated by other (unknown) mechanisms that repress transcription under the conditions applied here.

To get an indication whether the genes affected in the *codY* mutant were under direct or indirect control of CodY, the upstream regions of a number of them were tested for their ability to complex with purified H6-CodY (Fig. 2). Inspection of the DNA sequences did not initially reveal common motifs. A more comprehensive bioinformatics

approach using the upstream regions of only those genes that were derepressed to the highest extent led to the identification of an inversely repeated motif, AATTTTCWGAAAATT, that was present in most of the sequences constituting the data set. This motif (designated as CodY-box) shows homology to the upstream half-site of a palindromic sequence known to be important in *oppD* regulation (17). That study reported, among others, the analysis of several mutants that showed distorted CodY binding and regulation. Of these mutants, the ones that were most affected in *opp* regulation contained deletions and base pair substitutions within the stretch of nucleotides containing the CodY-box identified here. Here we show that this motif functions as an operator site for CodY. Multiple copies of the CodY-box are present in the intergenic regions of some transcriptional units, which might serve regulatory purposes; they could increase the affinity of CodY resulting in stronger repression. Similarly, orientation and similarity of the motif with its consensus sequence, of which the nucleotides at positions 3, 6, 7, 9, 10, and 13 are most strongly conserved (Fig. 3), might also contribute to the strength of regulation of a target gene by CodY. We show that the position of the DNA motif is important for effective regulation, as a 5-bp difference in variation of the distance of the box relative to the promoter determines whether the downstream gene is CodY-regulated (Fig. 6). As a difference of 5 bp equals half a helical turn, it places the CodY-binding site on the opposite side of the DNA helix. These experiments show that repression by CodY might be helix face-dependent. However, we cannot exclude the possibility that loss of CodY-mediated repression is caused by less efficient binding of the CodY protein to its target DNA sequence. Most DNA-binding proteins preferentially bind either to the major or minor groove in DNA. Introduction of half a helical turn of DNA would place the CodY-binding site in the opposite groove, which could negatively affect the efficiency of binding. X-ray crystallographic studies or DNA binding studies using interfering drugs would be helpful to get a better understanding of the molecular mechanisms of CodY binding.

A well-conserved copy of the CodY-box was found about 80 bp upstream of the *L. lactis codY* start codon. Using *in vitro* DNA binding assays and a P*codY-lacZ* fusion, we show that CodY binds to the upstream region of its gene *in vitro* and represses its own synthesis *in vivo* (Figs. 1 and 7). As in *B. subtilis* (21), expression of *L. lactis codY* is highest in the exponential phase of growth and decreases when cells enter the stationary phase. Although *B. subtilis codY* expression seems to be derepressed in minimal media lacking amino acid sources (20), it remains to be elucidated whether *B. subtilis codY* is also subjected to auto-regulation, especially because no obvious derivate of the CodY-box could be identified in its upstream region (supplemental Table 4). Auto-regulation provides an additional level of regulation of the CodY regulon, next to that exerted by BCAAs through modulation of the activity of CodY. In *B. subtilis*, GTP, as an alternative for auto-regulation, could provide this additional level of regulation. The lactococcal P*codY-lacZ* fusion construct was used to show that transcription of *codY* in the WT strain is low in media containing an excess of amino acids and peptides (in the form of casitone). Transcription driven from P*codY* is derepressed when the concentration of casitone is lowered (data not shown), which fits well with the results presented in Fig. 7, where the presence of the BCAA Ile was required for binding of lactococcal H6-CodY to the *codY* promoter fragment. A decrease in the basal activity of the *codY* promoter when cells approach the end of exponential growth, together with a concomitant relief of BCAA acid-mediated repression by CodY protein of its own transcription, might ensure that a certain amount of CodY remains present, allowing the cell to rapidly respond to changes in BCAA availability.

It has been shown recently that lactococcal CodY is able to modulate expression of *B. subtilis dpp*, encoding the dipeptide permease operon, in a *B. subtilis codY* mutant (18). Thus, both *B. subtilis* and *L. lactis* CodY must be able to recognize similar sequences, which was anticipated as their DNA binding domains are highly conserved (15). Other studies in *B. subtilis* have identified regions important for medium-dependent regulation of and/or for CodY binding to the promoter region of this gene (21, 22). Interaction of purified *B. subtilis* CodY with the upstream region of *dpp* was affected by several mutations in a short region close to its transcription start site. At the time it was unknown that this region contains a sequence (AATATTCATAATTTA) that resembles the CodY-box identified in our current study and forms part of a high affinity site for *B. subtilis* CodY (64). These binding studies showed that, with increasing concentrations of CodY, the protein was able to bind to several low affinity sites as well. Our footprinting data suggest that lactococcal CodY binding to the upstream regions of *gltA*, *serC*, and *codY* might occur in a similar manner. In addition to the region containing the CodY-box, multiple areas that are protected against DNase I activity can be distinguished, which might indicate that CodY can also bind to multiple sites in these promoters. As deletion of the CodY-box in P*codY* resulted in total loss of protection (Fig. 7), CodY might need a high affinity binding site, formed by the CodY-box, to be able to bind to other low affinity sites when intracellular levels of CodY protein or BCAAs are low.

Both deletion and mutational analyses of the operator region of the *B. subtilis* histidine utilization operon (*hut*) showed that an AT-rich stretch in the area from +10 to +24 relative to the transcription start site is required for amino acid-mediated regulation exerted by CodY (65, 66). As for the *B. subtilis dpp* operon (see above), these results can now be explained because the identified region overlaps with the CodY-box (AGTTATCAGAATTTT) found in this study. As we show here that CodY-boxes are present in several Gram-positive bacteria, it is tempting to speculate that this motif serves as a general CodY binding and regulatory site.

The CodY-box could not be discerned in the upstream regions of all the differentially expressed genes (*e.g. pepN* and *hisC*) by the bioinformatics tools used in this study. However, close inspection of the *pepN* upstream region did reveal a putative CodY-box (AATTTTCTATTCAAT). As this box is much less conserved than the ones present upstream of transcriptional units that were strongly derepressed upon functional removal of *codY* (TABLE THREE), it might explain why expression of this well known CodY target was not found to be significantly changed in our DNA microarray experiments. Absence of a CodY-box upstream of a gene affected by the *codY* mutation could indicate that its change in expression is an indirect effect of the mutation. Alternatively, it could be that other sequences play a role in the recognition and regulation of these genes by CodY. Transcriptional regulation of the CodY-dependent genes required for production of the proteinase PrtP of *L. lactis* SK11 has been investigated previously (16, 67). A mutational analysis of the intergenic region between *prtP* and *prtM* pinpointed an IR other than the CodY-box as being required for nitrogen-dependent regulation of the activity of the promoter. Previous studies in *B. subtilis* have not pinpointed a consensus sequence for CodY binding, and it has been proposed that CodY might recognize and bind a topological structure formed by AT-rich DNA (22). Such structures might be enhanced by the presence of an IR. This would also explain why regions of dyad symmetry with no apparent sequence homology were found to coincide with CodY binding to the *B. subtilis ilvB* (19) and *citB* promoters (29). Perhaps, the presence of a specific DNA structural motif could bypass the need of a CodY-box for CodY to

bind to its targets. The presence of such a DNA topology together with a high affinity binding site formed by the CodY-box might then result in maximum repression by CodY.

Acknowledgments—We are grateful to Aldert Zomer for the skillful assistance in the in silico analyses; Maarten Groeneveld for technical assistance in determining the expression patterns of ctrA and asnB; and Jan Sikkema, Ellen Looijesteijn, and Ingeborg Boels for helpful discussions.

REFERENCES

1. Godon, J. J., Delorme, C., Bardowski, J., Chopin, M. C., Ehrlich, S. D., and Renault, P. (1993) *J. Bacteriol.* **175,** 4383–4390
2. Chopin, A. (1993) *FEMS Microbiol. Rev.* **12,** 21–37
3. Delorme, C., Godon, J. J., Ehrlich, S. D., and Renault, P. (1993) *J. Bacteriol.* **175,** 4391–4399
4. Tynkkynen, S., Buist, G., Kunji, E., Kok, J., Poolman, B., Venema, G., and Haandrikman, A. (1993) *J. Bacteriol.* **175,** 7523–7532
5. Kunji, E. R., Mierau, I., Hagting, A., Poolman, B., and Konings, W. N. (1996) *Antonie Leeuwenhoek* **70,** 187–221
6. Kok, J., and Buist, G. (2003) *Genetics of Lactic Acid Bacteria* (Wood, B., and Warner, P. eds) pp. 189–223, Kluwer Academic Publishers, New York
7. Foucaud, C., Kunji, E. R., Hagting, A., Richard, J., Konings, W. N., Desmazeaud, M., and Poolman, B. (1995) *J. Bacteriol.* **177,** 4652–4657
8. Hagting, A., Kunji, E. R., Leenhouts, K. J., Poolman, B., and Konings, W. N. (1994) *J. Biol. Chem.* **269,** 11391–11399
9. Kunji, E. R., Mierau, I., Poolman, B., Konings, W. N., Venema, G., and Kok, J. (1996) *Mol. Microbiol.* **21,** 123–131
10. Lamarque, M., Charbonnel, P., Aubel, D., Piard, J. C., Atlan, D., and Juillard, V. (2004) *J. Bacteriol.* **186,** 6492–6500
11. Sanz, Y., Lanfermeijer, F. C., Renault, P., Bolotin, A., Konings, W. N., and Poolman, B. (2001) *Arch. Microbiol.* **175,** 334–343
12. Sanz, Y., Toldra, F., Renault, P., and Poolman, B. (2003) *FEMS Microbiol. Lett.* **227,** 33–38
13. Guédon, E., Renault, P., Ehrlich, S. D., and Delorme, C. (2001) *J. Bacteriol.* **183,** 3614–3622
14. Marugg, J. D., Meijer, W., van Kranenburg, R., Laverman, P., Bruinenberg, P. G., and de Vos, W. M. (1995) *J. Bacteriol.* **177,** 2982–2989
15. Guédon, E., Serror, P., Ehrlich, S. D., Renault, P., and Delorme, C. (2001) *Mol. Microbiol.* **40,** 1227–1239
16. Gajic, O. (2003) *Relationships between MDR Proteins, Bacteriocin Production and Proteolysis in Lactococcus lactis.* Ph.D. thesis, University of Groningen, Groningen, The Netherlands
17. den Hengst, C. D., Curley, P., Larsen, R., Buist, G., van Sinderen, D., Kuipers, O. P., and Kok, J. (2005) *J. Bacteriol.* **187,** 512–521
18. Petranovic, D., Guedon, E., Sperandio, B., Delorme, C., Ehrlich, D., and Renault, P. (2004) *Mol. Microbiol.* **53,** 613–621
19. Shivers, R. P., and Sonenshein, A. L. (2004) *Mol. Microbiol.* **53,** 599–611
20. Molle, V., Nakaura, Y., Shivers, R. P., Yamaguchi, H., Losick, R., Fujita, Y., and Sonenshein, A. L. (2003) *J. Bacteriol.* **185,** 1911–1922
21. Slack, F. J., Serror, P., Joyce, E., and Sonenshein, A. L. (1995) *Mol. Microbiol.* **15,** 689–702
22. Serror, P., and Sonenshein, A. L. (1996) *Mol. Microbiol.* **20,** 843–852
23. Fisher, S. H. (1999) *Mol. Microbiol.* **32,** 223–232
24. Fisher, S. H., Rohrer, K., and Ferson, A. E. (1996) *J. Bacteriol.* **178,** 3779–3784
25. Wray, L. V., Jr., Ferson, A. E., and Fisher, S. H. (1997) *J. Bacteriol.* **179,** 5494–5501
26. Débarbouillé, M., Gardan, R., Arnaud, M., and Rapoport, G. (1999) *J. Bacteriol.* **181,** 2059–2066
27. Shivers, R. P., and Sonenshein, A. L. (2005) *Mol. Microbiol.* **56,** 1549–1559
28. Tojo, S., Satomura, T., Morisaki, K., Deutscher, J., Hirooka, K., and Fujita, Y. (2005) *Mol. Microbiol.* **56,** 1560–1573
29. Kim, H. J., Kim, S. I., Ratnayake-Lecamwasam, M., Tachikawa, K., Sonenshein, A. L., and Strauch, M. (2003) *J. Bacteriol.* **185,** 1672–1680
30. Bergara, F., Ibarra, C., Iwamasa, J., Patarroyo, J. C., Aguilera, R., and Marquez-Magana, L. M. (2003) *J. Bacteriol.* **185,** 3118–3126
31. Inaoka, T., Takahashi, K., Ohnishi-Kameyama, M., Yoshida, M., and Ochi, K. (2003) *J. Biol. Chem.* **278,** 2169–2176
32. Serror, P., and Sonenshein, A. L. (1996) *J. Bacteriol.* **178,** 5910–5915
33. Terzaghi, B. E., and Sandine, W. E. (1975) *Appl. Microbiol.* **29,** 807–813
34. Kuipers, O. P., de Jong, A., Baerends, R. J. S., van Hijum, S. A. F. T., Zomer, A. L., Karsens, H. A., den Hengst, C. D., Kramer, N. E., Buist, G., and Kok, J. (2002) *Antonie Leeuwenhoek* **82,** 113–122
35. van Hijum, S. A. F. T., de, Jong, S., Baerends, R. J. S., Karsens, H. A., Kramer, N. E., Larsen, R., den Hengst, C. D., Albers, C. J., Kok, J., and Kuipers, O. P. (2005) *BMC. Genomics* **6,** 77
36. van Hijum, S. A. F. T., Garcia De La Nava, J., Trelles, O., Kok, J., and Kuipers, O.P. (2003) *Appl. Bioinformatics.* **2,** 241–244
37. Baldi, P., and Long, A. D. (2001) *Bioinformatics.* **17,** 509–519
38. Bailey, T. L., and Elkan, C. (1994) *Proc. Int. Conf. Intell. Syst. Mol. Biol.* **2,** 28–36
39. Baerends, R. J. S., Smits, W. K., de Jong, A., Hamoen, L. W., Kok, J., and Kuipers, O. P. (2004) *Genome Biol.* **5,** R37
40. Bolotin, A., Wincker, P., Mauger, S., Jaillon, O., Malarme, K., Weissenbach, J., Ehrlich, S. D., and Sorokin, A. (2001) *Genome Res.* **11,** 731–753
41. Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F., Danchin, A., *et al.* (1997) *Nature* **390,** 249–256
42. Hoskins, J., Alborn, W. E., Jr., Arnold, J., Blaszczak, L. C., Burgett, S., DeHoff, B. S., Estrem, S. T., Fritz, L., Fu, D. J., Fuller, W., Geringer, C., Gilmour, R., Glass, J. S., Khoja, H., Kraft, A. R., Lagace, R. E., LeBlanc, D. J., Lee, L. N., Lefkowitz, E. J., Lu, J., Matsushima, P., McAhren, S. M., McHenney, M., McLeaster, K., Mundy, C. W., Nicas, T. I., Norris, F. H., O'Gara, M., Peery, R. B., Robertson, G. T., Rockey, P., Sun, P. M., Winkler, M. E., Yang, Y., Young-Bellido, M., Zhao, G., Zook, C. A., Baltz, R. H., Jaskunas, S. R., Rosteck, P. R., Jr., Skatrud, P. L., and Glass, J. I. (2001) *J. Bacteriol.* **183,** 5709–5717
43. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
44. Leenhouts, K. J., Kok, J., and Venema, G. (1991) *J. Bacteriol.* **173,** 4794–4798
45. Holo, H., and Nes, I. F. (1995) *Methods Mol. Biol.* **47,** 195–199
46. Sanders, J. W., Venema, G., Kok, J., and Leenhouts, K. (1998) *Mol. Gen. Genet.* **257,** 681–685
47. Leenhouts, K., Bolhuis, A., Venema, G., and Kok, J. (1998) *Appl. Microbiol. Biotechnol.* **49,** 417–423
48. Maguin, E., Duwat, P., Hege, T., Ehrlich, D., and Gruss, A. (1992) *J. Bacteriol.* **174,** 5633–5638
49. Larsen, R., Buist, G., Kuipers, O. P., and Kok, J. (2004) *J. Bacteriol.* **186,** 1147–1157
50. Israelsen, H., Madsen, S. M., Vrang, A., Hansen, E. B., and Johansen, E. (1995) *Appl. Environ. Microbiol.* **61,** 2540–2547
51. Goupil-Feuillerat, N., Cocaign-Bousquet, M., Godon, J. J., Ehrlich, S. D., and Renault, P. (1997) *J. Bacteriol.* **179,** 6285–6293
52. Goupil-Feuillerat, N., Corthier, G., Godon, J. J., Ehrlich, S. D., and Renault, P. (2000) *J. Bacteriol.* **182,** 5399–5408
53. Godon, J. J., Chopin, M. C., and Ehrlich, S. D. (1992) *J. Bacteriol.* **174,** 6580–6589
54. Chambellon, E., and Yvon, M. (2003) *Appl. Environ. Microbiol.* **69,** 3061–3068
55. Yvon, M., Chambellon, E., Bolotin, A., and Roudot-Algaron, F. (2000) *Appl. Environ. Microbiol.* **66,** 571–577
56. Crow, V. L., and Thomas, T. D. (1982) *J. Bacteriol.* **150,** 1024–1032
57. Kanehisa, M., Goto, S., Kawashima, S., and Nakaya, A. (2002) *Nucleic Acids Res.* **30,** 42–46
58. Wang, H., Baldwin, K. A., O'Sullivan, D. J., and McKay, L. L. (2000) *J. Dairy Sci.* **83,** 1912–1918
59. Overweg, K., Kerr, A., Sluijter, M., Jackson, M. H., Mitchell, T. J., De Jong, A. P., De, G. R., and Hermans, P. W. (2000) *Infect. Immun.* **68,** 4180–4188
60. Chaussee, M. A., Callegari, E. A., and Chaussee, M. S. (2004) *J. Bacteriol.* **186,** 7091–7099
61. Morishita, T., and Yajima, M. (1995) *Biosci. Biotechnol. Biochem.* **59,** 251–255
62. Lapujade, P., Cocaign-Bousquet, M., and Loubiere, P. (1998) *Appl. Environ. Microbiol.* **64,** 2485–2489
63. Yue, H., Eastman, P. S., Wang, B. B., Minor, J., Doctolero, M. H., Nuttall, R. L., Stack, R., Becker, J. W., Montgomery, J. R., Vainer, M., and Johnston, R. (2001) *Nucleic Acids Res.* **29,** 41
64. Joseph, P., Ratnayake-Lecamwasam, M., and Sonenshein, A. L. (2005) *J. Bacteriol.* **187,** 4127–4139
65. Wray, L. V., Jr., and Fisher, S. H. (1994) *J. Bacteriol.* **176,** 5466–5473
66. Eda, S., Hoshino, T., and Oda, M. (2000) *Biosci. Biotechnol. Biochem.* **64,** 484–491
67. Marugg, J. D., van Kranenburg, R., Laverman, P., Rutten, G. A., and de Vos, W. M. (1996) *J. Bacteriol.* **178,** 1525–1531
68. Gasson, M. J. (1983) *J. Bacteriol.* **154,** 1–9